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DOI: https://doi.org/10.30802/aalas-jaalas-17-000118

Journal of the American Association for Laboratory Animal Science
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Vol 57, No 4
July 2018
Pages 368–375

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This study investigated the analgesic activity of tramadol in female C57BL/6J mice by using a single subcutaneous injection (25 mg/kg) of tramadol combined with the same dose given in drinking water for 24 h. We then evaluated the pharmacokinetics of tramadol and its active metabolite O-demethyltramadol (M1). To evaluate pain and analgesic efficacy, we performed clinical and behavioral assessment, burrowing tests, and activity analysis and measured body weight, food and water intake in mice that were untreated (control) or underwent analgesia only (T); anesthesia and surgery (AS); or anesthesia, surgery, and analgesia (AS+T). The plasma concentration of tramadol decreased rapidly whereas, for more than 18 h, the M1 level remained stable and above its minimal analgesic concentration for humans. Total food and water intake over 24 h was comparable among all groups. Although T mice consumed tramadol-treated water in sufficient amount and frequency, AS and AS+T animals showed decreased drinking frequency during the first 4 h after surgery. Compared with control and T groups, composite pain scores and burrowing latencies increased significantly in both AS and AS+T mice after surgery, suggesting postsurgical pain. However, AS and AS+T mice did not differ significantly after surgery. In conclusion, although naïve animals ingested a sufficient amount of the drug and plasma levels appeared sufficiently high, mice markedly reduced water intake immediately after surgery. Consequently, even in combination with an initial drug injection, the subsequent voluntary tramadol intake was insufficient to reduce signs of postsurgical pain significantly after laparotomy.

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Effective pain treatment in laboratory animals undergoing surgical procedures is essential from an ethical standpoint, because pain and stress affect animal welfare detrimentally. In addition, pain may entail negative physiologic consequences, which can become significant confounders in research, by increasing inter- and intraanimal variation and potentially leading to inaccurate results.[10,12,14]

Continuous refinement of pain management in laboratory mice involves increasing the spectrum of analgesics available for this species; optimizing analgesic dose regimens (dose, frequency, and duration); implementing new methods of administration (sustain-released formulations, voluntary ingestion); and developing new methods for pain assessment.

Tramadol is a centrally acting synthetic opioid, structurally related to morphine and codeine[2,18,33] and used for the treatment of moderate to severe pain, both acute and chronic, in various species, including humans.[21] Tramadol has some selectivity for the μ receptor and binds weakly to κ and δ receptors; furthermore, tramadol activates the monoaminergic system, inhibiting neuronal reuptake of serotonin (5-hydroxytryptamine) and noradrenaline (norepinephrine).[3,27] The clinical efficacy of tramadol is highly related to its first metabolite, O-demethyltramadol hydrochloride (M1), which has 200-fold higher affinity for μ receptors and as much as 6-fold higher analgesic potency than tramadol itself.[25,30] Because of its 'double' mechanism of action, tramadol at clinical doses does not induce respiratory depression or hemodynamic changes, which are common to other opioids with high μ-receptor activity.[9,21,26] Moreover, tramadol is an affordable and non-controlled substance in many countries, thus facilitating its use.

Published data regarding the pharmacokinetics, dose regimens, and analgesic efficacy of tramadol in mice are few, and the evidence available is, at best, controversial. One study[24] demonstrated that tramadol ameliorates cyclophosphamide-induced bladder-pain-related behaviors in mice, whereas another[31] found that tramadol did not reduce symptoms of postsurgical pain after abdominal laparotomy in mice. However, in the laparotomy study,[31] the drug was injected every 12 h, which is likely to have been insufficient for effective pain relief.

The administration of drugs through voluntary ingestion in laboratory rodents has gained popularity in recent years, because this method reduces the handling stress that accompanies intraperitoneal and subcutaneous injections—hitherto the most common routes used in mice.[7,8,29] Voluntary consumption of buprenorphine, for example, in food items and drinking water results in high serum concentrations with significant antinociception and a longer duration of action in rats than the widely recommended subcutaneous route.[17] Conversely, in mice, buprenorphine self-administration through drinking water might lead to variable and low blood concentrations of the drug during the animals' resting phase, thus perhaps necessitating additional doses to reach therapeutically effective drug levels.[28] However, studies from our laboratory have shown that self-administration of 25 mg/kg tramadol in drinking water resulted in high and stable plasma levels of drug in naïve C57BL/6J mice throughout the consumption time (including the animals' resting phase), with levels decreasing rapidly after administration ceased.[4]

In the present study, we hypothesized that a subcutaneous injection of tramadol (25 mg/kg) followed by voluntary ingestion of tramadol in drinking water (25 mg/kg) over 24 h would

Received: 22 Sep 2017. Revision requested: 24 Oct 2017. Accepted: 20 Dec 2017.
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provide effective postoperative analgesia in C57BL/6J mice undergoing unilateral sham embryo transfer surgery. We assessed the efficacy of the chosen analgesic protocol by 1) evaluating clinical and behavioral markers of pain; 2) measuring body weight and food and water intake; 3) performing burrowing tests; and 4) generating a pharmacokinetic profile.

Materials and Methods

Ethics statement. The Cantonal Veterinary Office (Zurich, Switzerland) approved the animal housing and experimental protocols under license no. ZH 181/2012; procedures were in accordance with Swiss Animal Protection Law and conform to European Directive 2010/63/EU of the European Parliament and the Council on the Protection of the Animals used for Scientific Purposes and to the *Guide for the Care and Use of Laboratory Animals*, 8th edition (Council 2011).[13]

Animals and standard housing conditions. Female C57BL/6J mice (n = 64; age, 6 to 8 wk; weight, 18 to 22 g) were obtained from a commercial supplier (Charles River, L'Arbresle, France). Female animals were used for comparability with previous studies (for example, references 14 and 26). The animals' health status was monitored by a health surveillance program according to the Federation of European Laboratory Animal Science Associations (FELASA) guidelines. The mice were free of all viral, bacterial, and parasitic pathogens listed in the FELASA recommendations[22] (mouse hepatitis virus, mouse rotavirus, murine norovirus, minute virus of mice, mouse parvovirus, Theiler murine encephalomyelitis virus, lymphocytic choriomeningitis virus, mouse adenovirus type 1 [FL], mouse adenovirus type 2 [K87], mousepox [ectromelia] virus, pneumonia virus of mice, reovirus type 3, Sendai virus, *Helicobacter* spp., *Pasteurella pneumotropica*, β-hemolytic streptococci, *Streptococcus pneumonia*, *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium kutscheri*, *Mycoplasma pulmonis*, *Salmonella* spp., *Streptobacillus moniliformis*, and endo- and ectoparasites).

Mice were housed in groups of 4 to 8 for 3 wk prior to the beginning of the experimental phase. The animals were kept in conventional transparent plastic cages (Eurotype III, Techniplast, Hohenpeißenberg, Germany) with autoclaved dust-free sawdust bedding (80 to 90 g per cage; LTE E-001, Abedd, Indulab, Gams, Switzerland), hay (8 to 12 g per cage; Winzeler, Affoltern am Albis, Switzerland), and cardboard shelters (Ketchum Manufacturing, Brockville, Ontario, Canada). They were fed a pelleted and extruded mouse diet (no. 3436, Provimi Kliba, Kaiseraugst, Switzerland) without restriction and had unrestricted access to drinking water. The room was maintained on a 12:12-h light:dark cycle with artificial light (approximately 40 lx in the cage). The temperature was 21 ± 1 °C, with a relative humidity of 55% ± 10%. Animals were handled by the tail. Disturbances (that is, unrelated experimental procedures) were not allowed in the housing room.

Analgesic protocols. The analgesic protocol used was tramadol hydrochloride (25 mg/kg SC; Tramal 100 injection, Grunenthal, Stolberg, Germany) administered 10 to 20 min before surgery, followed by tramadol in drinking water (25 mg/kg), beginning 1 h after the injection and continuing for 24 h. Shortly before injection, tramadol was diluted in sterile NaCl 0.9% (B Braun Medical, Sempach, Switzerland) so that the injection volume was 0.04 mL per 10 g body weight. Bottles containing tramadol in drinking water were prepared shortly before surgery and had a final concentration of 0.2 mg/mL.

Experimental design, experimental animal housing conditions, and data acquisition. Mice were randomly (complete and blinded randomization) assigned to 1 of the 2 behavioral assessment settings and 1 of 4 experimental groups (n = 8 per setting and group): controls (naïve, no manipulation); T group, (tramadol injection followed by tramadol in drinking water for 24 h); AS group, mice underwent surgery and anesthesia without analgesic treatment but received an injection of saline; or AS+T group, mice underwent anesthesia, surgery, and analgesia with tramadol as described earlier.

Behavioral assessment involved 2 experimental settings and was performed after surgery or respective control procedures: 1) burrowing test and clinical assessment in a standard cage,[16] or 2) automated activity analysis and measurement of food and water intake and body weight in an observation cage. Mice were housed individually in one of these settings for 2 d before and 1 d after experimental intervention.

For the surgical procedure, the experiments began 60 to 90 min before the start of the dark phase, the active phase of the mice. The fur at the surgical site was clipped, and the mice received a subcutaneous injection of tramadol or saline. At 10 to 20 min after injection, the animals were transferred in individual transport cages to an operating theater and anesthetized with sevoflurane (Sevorane, Abbot, Baar, Switzerland) as monoanaesthesia. The anesthetic gas was provided by using a rodent inhalation anesthesia system (Provet, Lyssach, Switzerland); oxygen was the carrier gas. The mice were placed on a warming mat (TP500, Gaymar, Orchard Park, NY) set at 39 ± 1 °C to ensure constant body temperature, and anesthesia was induced and maintained through a nose mask (8% and 6% sevoflurane at 600 mL/min gas flow, respectively). The surgical site was disinfected with chlorhexidine.

Mice in surgery groups underwent a one-sided sham embryo transfer procedure under aseptic technique. The skin in the mouse's left flank was incised vertically approximately 2 fingers cranial to the tail root, with a length of approximately 0.5 cm. The subcutis was cut and the abdominal muscle was incised. The underlying fat was lifted, exposing the ovary. The ovary was then elevated, thus stretching the ligaments to mimic the embryo transfer procedure. After relocation of the ovary, the incision in the abdominal muscle wall was closed with absorbable suture (6-0 polyglycolic acid suture, Ethicon, Norderstedt, Germany), and the skin was closed by using skin staples (Precise, 3M Health Care, St Paul, MN). Surgery was completed within 3 to 4 min, and anesthesia lasted 6 to 8 min. Mice (while moving freely in their transport cages) were allowed to recover for 30 to 40 min in a warming cabinet set at 32 °C. Then they were returned to their home cages.

Setting 1. *Clinical and behavioral assessment*. For clinical investigation and behavior-based pain assessment, mice were placed individually in a small acrylic observation chamber (10 × 10 × 10 cm), where each animal was observed for 4 min. Observations took place at 1 and 4 h after tramadol or saline injection, or at comparable time points for control animals. Scoring was performed according to a system that documented the general condition, indicators of abdominal pain, and changes in facial expression.[16] Clinical signs were then converted into numerical scores according to a scoring system (Figure 1) and summed to obtain a composite score for each animal. Investigators performing behavioral testing (PJ, RE) were blinded for treatment groups.

Burrowing test. The burrowing test uses the natural burrow digging and cleaning behavior of mice and determines spontaneous burrowing activity. Assessing burrowing latencies can be used as a simple method to assess postsurgical impairment and pain in mice. Standard cages contained a pellet-filled water bottle in one corner of the cage.[15] Cages were videorecorded

Parameter	Signs	Score
Orbital tightening	Narrowing of the orbital area, eyelids tightly closed, or squinting	0, not present 1, less than 50% closed 2, 50% closed or more
Ear position	Ears pulled back or rotated outward or backward, away from the face; space between the ears may appear wider	0, not present 1, rotated 2, nearly flat against head
Stretch	Abdomen pushed to floor, hindpaws stretched backward	0, not present
Press	Abdomen pushed to floor	2, 3 events or more 1, fewer than 3 events
Posture	Hunched, arched back, crouched	
Spontaneous behavior	Sudden movement, backward movement, transient involuntary muscular contraction of any body part, kicking with hindpaws, licking or biting the wound, highly aggressive, vocalization	0, not present 1, present
Coat condition	Ruffled, dirty, unkempt, piloerection, hair loss (alopecia)	
Eyes	Discharge	
Body condition	Sunken flanks, swollen areas, ascites	
Wound	Dirty, bloody, uncleaned, signs of self-injury, signs of inflammation or necrosis (that is, unusual color [for example, red, pale] or swollen)	
Movement	Apathetic, sedated, slower, crawling, immobile, lameness, tiptoeing	

Figure 1. Scoring system for clinical investigation and behavior-based pain assessment. In total, a maximal score of 15 can be reached. Each mouse was observed for 4 min.

from above by using infrared-sensitive cameras (Ikegami, Tokyo, Japan). The latency of each animal to burrow (the removal of more than 3 pellets within 10 s) was assessed by analyzing videorecordings taken during the first 12 h after the return of the animal to its home cage (1 h after completion of surgery), or at a comparable time point for control and T animals. Investigators analyzing the videorecordings (PJ, RE) were blinded for treatment groups.

Setting 2. *Activity analysis*. Observation cages were videorecorded for 24 h, starting 1 h after the end of surgery or at a comparable time point, from above by using infrared-sensitive cameras for analysis of home-cage activity by using automated tracking software (EthoVision XT 7, Noldus Information Technology, Wageningen, Netherlands). The distance (in centimeters) over which the center point of the animal moved was assessed to measure static behaviors as well as horizontal locomotion.

***Body weight and food and water intake*.** The animals, food pellets, and water bottles were weighed directly before and 24 h after the procedures. A blinded observer viewing the videotapes counted the drinking events during the first 4 h after the return of the mouse to its home cage, or at a comparable time point for control animals.

Serum levels and calculation of maximal plasma concentration. After a washout phase (at least 2 wk), 42 randomly selected mice were reused for establishing the pharmacokinetic profile of tramadol. The animals used for pharmacokinetic profiling were housed in groups of 8 in standard cages, as described earlier; 6 mice were used for each time point (1, 2, 4, 12, 18, 24, and 28 h after subcutaneous administration of tramadol, and continuous administration in drinking water). Mice underwent deep terminal anesthesia with sevoflurane, and blood was collected by cardiocentesis. Blood was centrifuged, and the plasma was stored at –20 °C until further analysis. Plasma concentrations of tramadol and M1 were determined by HPLC followed by tandem mass spectrometry at a commercial laboratory (Toxilab Ludwigsburg, Laboratory for Toxicology and Drug Evaluations, Ludwigsburg, Germany). Peak plasma concentration was calculated by using appropriate software (Phoenix WinNonlin Version 6.4; Pharsight Corp., CA).

Statistical analyses. Power calculation for group size determination was performed by using G*Power 3.1[5] Statistical analyses were performed by using Prism 6 (GraphPad Software, La Jolla, CA) and SPSS 23 (IBM, Armonk, NY). All data were tested for normal distribution and homogeneity of variance (Shapiro–Wilks and Levene tests). Means and SD were calculated for all parameters. Univariate ANCOVA were performed for body weight, changes in the weights of food pellets and water bottles (with corresponding baseline values as covariates) followed by post hoc tests (Bonferroni). Composite scores, as well as distance moved during 24 h of activity analyses, were evaluated through one-way ANOVA followed by post hoc tests (Bonferroni) to show significant differences between groups. Kaplan–Meier survival analysis was performed to examine the distribution of time to effect (latency to burrow). To test whether the latency to burrow differed between treatment groups, a log-rank significance test was performed. The threshold for significance in all statistical tests was set at a P value of 0.05.

Results

Setting 1. *Clinical assessment*. Observation of the mice revealed no physical complications from the surgical procedures performed (that is, open wounds) or of the analgesic treatment (that is, skin irritation at the injection site). During the observation time, none of the mice bit or manipulated their incision sites.

Composite scores differed significantly after 1 h ($P < 0.0001$, df = 3, F = 11.25) and 4 h ($P = 0.001$, df = 3, F = 6.992). Compared with control scores, composite score means were increased in all experimental groups (Figure 2). Animals that underwent

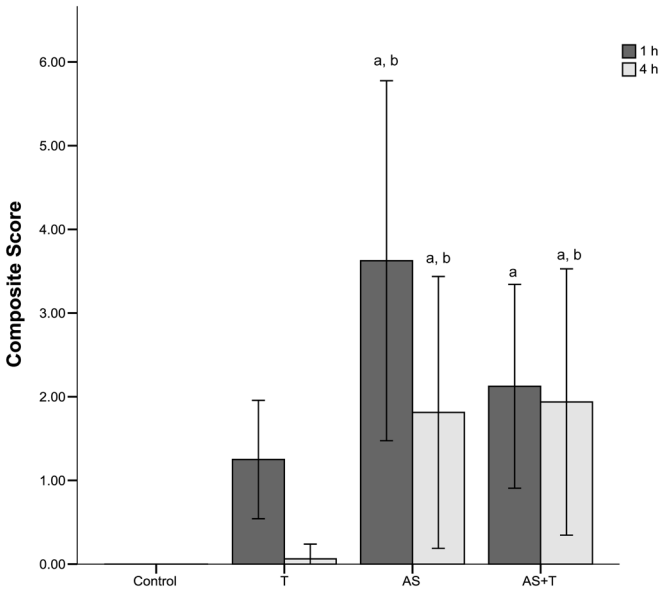

Figure 2. Composite scores (mean ± 1 SD) at 1 and 4 h after recovery from anesthesia (or at comparable time points in T and control mice). Compared with those in controls, composite scores were increased in all experimental groups. Mice that underwent surgery, with or without analgesic treatment, reached higher composite scores than control or T mice; at 1 h, these differences were significant between AS and AS+T groups compared with controls and between T and AS mice. At 4 h the scores of both the AS and AS+T groups differed significantly from the control and T group scores. Significantly ($P \leq 0.05$) different from [a]control or [b] T groups. All groups contained 8 mice. T, subcutaneous tramadol followed by tramadol in drinking water; AS+T, surgery and anesthesia and analgesia; AS, surgery and anesthesia (mice underwent anesthesia and surgery without analgesic treatment).

surgery, with or without analgesic treatment, achieved higher composite scores than those for control or T group scores, thus suggesting postsurgical pain. These differences were significant at 1 h for the comparison of AS and AS+T groups with controls ($P < 0.001$ and $P = 0.016$, respectively) and for T compared with AS ($P = 0.006$). At 4 h, the scores of both surgery groups were significantly greater than the control score (AS, $P = 0.022$; AS+T, $P = 0.012$) and the T group score (AS, $P = 0.028$; AS+T, $P = 0.016$).

Despite the lack of significant differences between the composite scores of groups that underwent surgery with or without analgesia, we noted some interesting, albeit nonsignificant, tendencies in the individual scoring units. For example, hunched posture and decreased fur condition occurred in all experimental groups except for control animals, whereas all animals that underwent surgery, regardless of analgesic treatment, showed increased orbital tightening and rotation of the ears. Stretching and pressing movements were more frequent in the AS group (that is, no analgesia).

By 4 h after surgery, the general appearance of the mice (fur, posture, and so forth) had normalized in all groups. Grooming was observed from 1 h onward, and all mice showed normal periods of activity followed by periods of resting.

Burrowing test. Burrowing latencies in control animals (7.5 ± 5.5 min) and after tramadol treatment alone (21.34 ± 17.14 min; $P = 0.0513$ compared with control) were short. Compared with control, burrowing latencies were prolonged significantly after surgery with and without analgesia (AS+T: 214.9 ± 78.3 min, $P = 0.0002$; AS: 191.9 ± 135.7 min, $P = 0.0015$), thus suggesting postsurgical pain. Burrowing latency did not differ between experimental groups, except for AS+T compared with T ($P = 0.0155$; Figure 3).

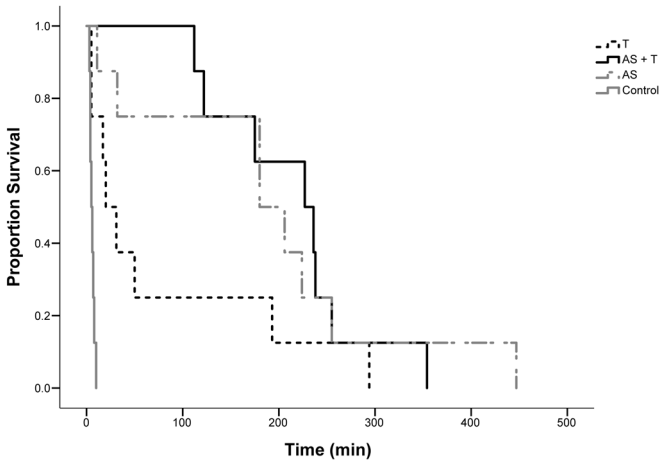

Figure 3. Survival analysis for burrowing latencies. The graph shows the survival percentage of mice that had not yet started to burrow as distributed over time. Burrowing latencies in control animals were short. Tramadol treatment alone increased latency to burrow non-significantly compared with control latency. Burrowing latencies were prolonged significantly after surgery with and without analgesia with tramadol compared with control latency. No significant differences were observed between experimental groups, except for AS+T compared with T. All groups contained 8 mice. T, analgesia with subcutaneous tramadol followed by tramadol in drinking water; AS+T, surgery and anesthesia and analgesia; AS, surgery and anesthesia (mice underwent anesthesia and surgery without analgesic treatment).

Setting 2. ***Activity analysis.*** Mean center point movement was comparable between groups that did not undergo surgery (control, 1477.49 ± 137.97 m; T, 1252.33 ± 81.18 m, respectively, $P = 0.18$), whereas groups undergoing surgery showed decreased activity. However, the decrease in mean distance was significant only for the AS+T group (1259.85 ± 67.89 m) compared with both the controls ($P < 0.001$) and T group ($P = 0.0002$).

Body weight and food and water intakes. Compared with the control group (17.7 ± 0.7 g before procedures compared with 17.9 ± 0.8 g afterward), all treated groups showed a decrease in body weight after experiments (T, 19.1 ± 0.8 g compared with 18.7 ± 0.7 g; AS, 20.6 ± 1.7 g compared with 20.1 ± 1.8 g; and AS+T, 20.1 ± 2.4 g compared with 19.4 ± 2.3 g). Treatment had a significant effect on experimental body weight after correction for baseline body weight ($F_{3, 25} = 3.985$, $P = 0.019$, partial $\eta^2 = 0.323$). Post hoc tests revealed a significant ($P = 0.018$) difference in body weight between the control and AS+T groups.

Food intake decreased slightly in all groups after experiments (control, 3.8 ± 0.3 g compared with 3.2 ± 0.3 g; T, 3.4 ± 0.4 g compared with 3.1 ± 0.4 g; AS, 3.8 ± 0.6 g compared with 3.1 ± 0.8 g; and AS+T, 3.4 ± 0.5 g compared with 2.3 ± 0.7g). Treatment had a significant effect on postprocedural food intake after correcting for baseline food intake ($F_{3, 27} = 3.729$, $P = 0.023$, partial $\eta^2 = 0.293$). Post hoc tests revealed a significant ($P = 0.04$) difference in food intake between the T and AS+T groups.

In most groups, water intake was nonsignificantly lower after experiments (control, 5.1 ± 0.9 g compared with 4.9 ± 0.8 g; T, 4.5 ± 0.3 g compared with 4.3 ± 0.5 g; AS, 5.0 ± 0.7 g compared with 4.8 ± 1.7 g; and AS+T, 4.5 ± 0.5 compared with 4.5 ± 2 g). After correction for baseline intake, treatment had no effect on water intake after procedures ($F_{3, 27} = 0.208$, $P = 0.890$, partial $\eta^2 = 0.023$).

Drinking event frequency during the first 4 h was comparable between control (20 ± 5 events) and T (20 ± 8 events) groups but appeared reduced in AS (6 ± 2 events) and AS+T (7 ± 3 events) groups (Figure 4).

Serum concentrations of tramadol and M1. In naïve mice, serum concentrations of tramadol were high (maximum, 670

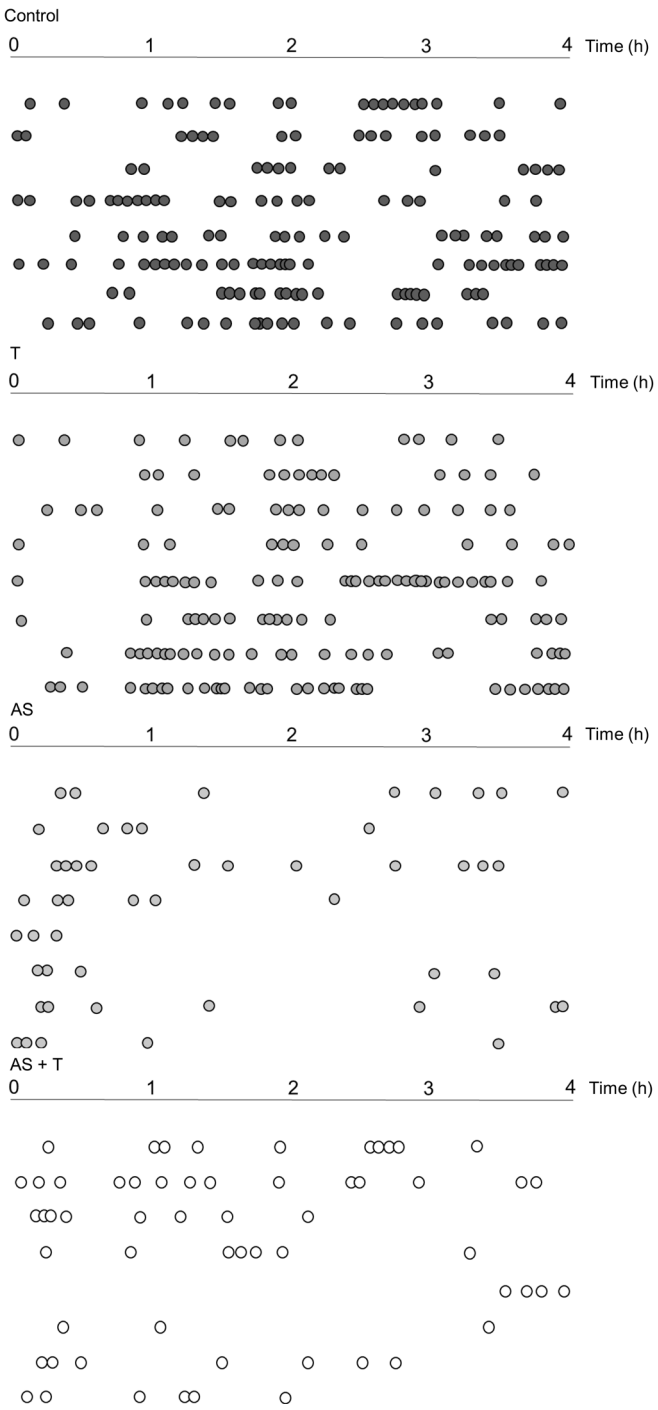

Figure 4. Drinking event frequency during the first 4 h was comparable between control and T groups but reduced in AS and AS+T groups. Each row of dots represents the drinking events for an individual mouse. Data are solely descriptive; no statistical analysis was applied. All groups contained 8 mice. T, analgesia with subcutaneous tramadol followed by tramadol in drinking water; AS+T, surgery and anesthesia and analgesia; AS, surgery and anesthesia (mice underwent anesthesia and surgery without analgesic treatment).

± 359 ng/mL) during the first hour of administration. The concentration of tramadol then decreased rapidly during the first 2 h after treatment to levels below the minimal analgesic concentration established in humans (100 ng/mL). In contrast, the highest serum concentrations of M1 occurred at 1 h after subcutaneous administration of tramadol (2460 ± 478 ng/mL) and remained above the minimal analgesic concentration for

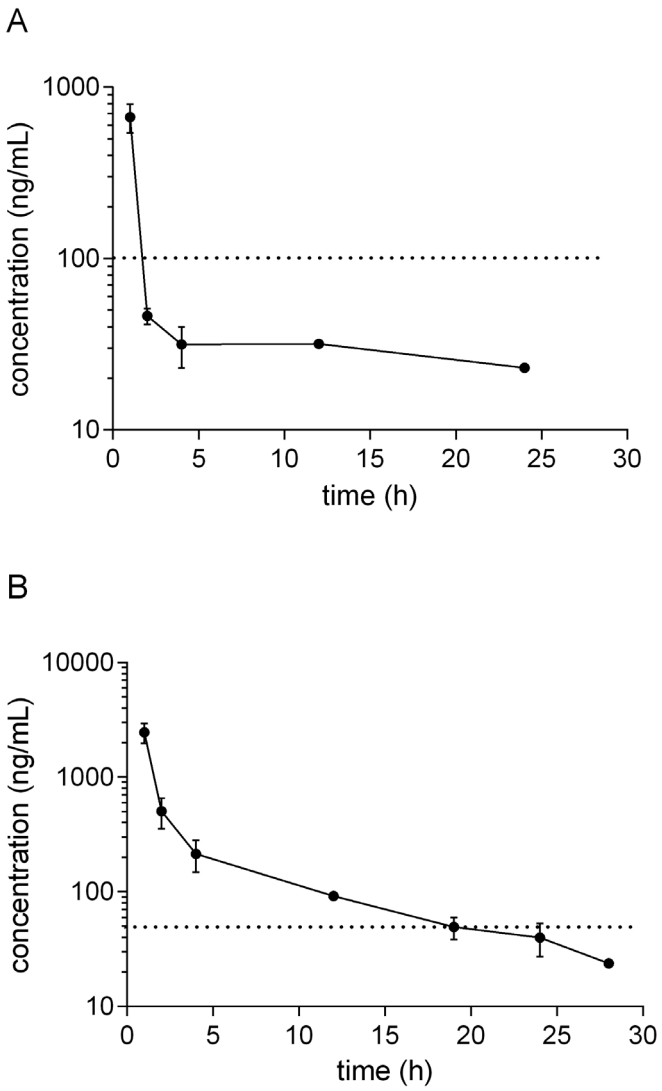

Figure 5. (A) Semilog plot of serum tramadol concentrations (mean ± 1 SD) over time after subcutaneous injection of 25 mg/kg tramadol followed by 25 mg/kg tramadol in drinking water for 24 h. The horizontal line represents the minimal analgesic concentration of tramadol in humans (100 ng/mL). (B) Semilog plot of serum M1 concentrations (mean ± 1 SD) over time after subcutaneous administration of 25 mg/kg tramadol followed by 25 mg/kg tramadol in drinking water for 24 h. The horizontal line represents the minimal analgesic concentration of M1 in humans (40 ng/mL) in humans.

humans (40 ng/mL)[20] until 18 h after tramadol administration began (Figure 5).

Discussion

The aim of the present study was to explore whether the combination of a single subcutaneous injection of tramadol (25 mg/kg) followed by voluntary ingested tramadol (25 mg/kg over 24 h in drinking water) offered reliable analgesic coverage in a clinical relevant mouse model of mild surgical pain.

Previous pharmacokinetic data from our lab indicated that presurgical injection of tramadol would be sufficient to provide perioperative analgesia for at least 1.5 to 2 h and that continuous postoperative analgesia would be achieved by providing tramadol in the drinking water.[4] The analgesic potency of tramadol is appropriate for mild to moderate pain.[21] The analgesic efficacy

of tramadol depends on the parent compound and on its first metabolite, which is as much as 6 times more potent than tramadol itself.[25,30] Although the serum concentrations of tramadol in our mice decreased rapidly, the levels of M1 remained high for 18 h in our study, and we, therefore, expect an analgesic effect from the tested treatment.

We chose the unilateral sham embryo-transfer surgery model, which elicits mild to moderate pain, based on ethical grounds—to avoid using a more severe surgical procedure in the light of unconfirmed analgesic efficacy—and in light of the spread and relevance of this intervention (laparotomy for embryo transfer) in female laboratory mice.[19,23] The recognition of mild to moderate pain in mice can be challenging because mice typically mask overt signs of pain or discomfort. We therefore used clinical monitoring tools as well as sensitive behavior-based pain indicators to assess pain and analgesic efficacy in the current study.[6,15,16,23,32] Even though the pain induced in our study was likely mild in nature, our behavior-based pain scoring methods detected a clear and significant effect of surgery. The observed decrease of pain signs at 4 h after surgery supports the expected duration of postsurgical pain in the laparotomy model used.

Home-cage observations revealed that mice that received tramadol but underwent no further experimental procedures willingly drank the calculated dose of tramadol during the 24-h time period (approximately 25 mg/kg). During the first 4 h of the experiment, this intake was frequent and regular and is therefore in agreement with the pharmacokinetic analyses mentioned. Nevertheless, even though total intake of water for 24 h remained stable in all experimental groups and oral analgesia administration was initiated during the activity phase of mice, when mice normally drink frequently and regularly,[28] drinking water intake in mice that underwent anesthesia and surgery was less regular and frequent during the immediate postsurgical phase, that is the first 4 h after surgery, compared with control and T groups. This effect might be due to the influence of surgery and anesthesia procedures on the animals' behavior. Because the first few hours after surgery are probably the period of highest pain levels, a reduction in drinking frequency and thus likely also in water intake, might interfere with the efficacy of an analgesia protocol, particularly one involving a short-acting drug like tramadol. Consequently, we observed signs of unrelieved pain, that is prolonged burrowing latencies and increased composite pain scores, not only in animals that underwent surgery without pain treatment but also in mice that received the combination treatment of injected and oral tramadol perioperatively.

Burrowing latency is increased by postsurgical pain in mice.[15] However, an increase in the latency to burrow has been also reported in animals treated with buprenorphine only,[16] due to increased locomotor activity, a well-known adverse effect of buprenorphine.[11] The effect of tramadol on burrowing activity was unlikely due to changes in general activity, given that the administration of tramadol in the absence of surgery did not alter 24-h home-cage locomotion. However, burrowing latency was increased significantly in both surgery groups, hinting that this tramadol dose failed to provide sufficient pain relief. Similarly, composite pain scores were significantly increased in both surgery groups compared with controls.

Monitored clinical signs were affected in all experimental groups, suggesting general effects of anesthesia, surgery, and tramadol itself on wellbeing. Whereas all mice that underwent surgery, regardless of analgesic treatment, showed approximately the same level of increased orbital tightening and rotation of the ears, mice in the AS group (that is, without analgesia) more frequently demonstrated stretching and pressing movements were observed more. These behavior results suggest the higher pain specificity of these assessment parameters compared with more general clinical signs. Although these behaviors, especially the press and stretch events, were too rare for statistical analysis, this observation suggests that the analgesic treatment had some effect on signs of pain specific for this model of surgery (abdominal laparotomy). Consequently, the tramadol protocol used may have achieved partial albeit insufficient pain relief in the tested model. We are unable to discriminate the effects of anesthesia on the behavioral results from the pain effects here, but other studies suggest that the effect of inhalation anesthesia on the read-out of the applied test can be controlled.[15,16]

The lack of significant differences in pain measures between the AS and AS+T groups may be due to the high interindividual variability in both surgery groups. In turn, this interindividual variability might at least partially be explained by the irregular and variable oral intake of tramadol after surgery. Alternatively, the dose we selected, although based on previous PK data,[4] might have been too low to reach an effective blood concentration of tramadol and M1 in mice. To date, target blood concentrations are known for humans only and may not be directly translatable to mice. The preemptive subcutaneous injection of 25 mg/kg tramadol before surgery has a presumable postsurgical duration of action of 2 to 4 h[4] and is therefore insufficient to bypass a potentially reduced intake or potential lack of efficacy of voluntary ingested tramadol. Moreover, the increased composite scores in the AS+T group at 1 h suggest that the subcutaneous injection might also have failed to sufficiently relieve acute postsurgical pain; this finding might again hint at the insufficient analgesic action of tramadol at the dose analyzed in our model.

In addition to the moderate effects of tramadol on the behavioral pain assessment parameters we used here, other side effects of opioids might be expected. For example, opioids frequently decrease water and food intake in rodents and consequently reduce body weight, an effect that has frequently been ascribed to buprenorphine[1] and one that might be detrimental in the postsurgical recovery period. In the present study, body weight and total food and water intake over 24 h were similar among all experimental groups and differed only slightly compared with control animals. This effect included animals that received tramadol but that did not undergo surgery. Therefore, tramadol shows probably less severe behavioral side effects than the popular buprenorphine.

In conclusion, the analgesia protocol chosen, consisting of a single subcutaneous injection of tramadol at 25 mg/kg followed by tramadol at 25 mg/kg in drinking water for 24 h does not appear to provide full or sufficient analgesic coverage for the treatment of mild to moderate postsurgical, abdominal pain. The lack of efficacy might be due to high interindividual variability in the intake of treated drinking water,[28] to the observed reduction of drinking frequency during the postsurgical phase, or to an insufficient dose. However, even though the differences were not significant, composite pain scores were slightly lower in tramadol-treated as compared with untreated operated mice. This result is promising and encourages the completion of additional studies to find better and more reliable tramadol protocols for mice.

Therefore, as next steps, it is necessary to establish the therapeutically effective dose of tramadol and its serum concentration in mice and to confirm that opioid-related side effects remain

low when doses are increased. Furthermore, because sexes and mouse strains and lines may differ in regard to nociception and in response to different analgesics, the analgesic efficacy of tramadol must be evaluated in males and different mouse strains before it is used routinely for postsurgical analgesia in mice.

Acknowledgments

We thank the Vet Service Team of F Hoffmann-La Roche, Robin Schneider, Hugo Battaglia, Mattea Durst, and the staff of the Central Biological Laboratory for their valuable support.

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